Serum Hormones, Follicular Fluid Steroids, Insulin-Like Growth Factors and Their Binding Proteins, and Ovarian IGF mRNA in Sheep with Different Ovulation Rates[1]

L. J. Spicer*,[2], S. E. Echternkamp†, E. A. Wong‡, D. T. Hamilton‡, and R. K. Vernon*

*Department of Animal Science, Oklahoma State University, Stillwater 74078,
†U.S. Meat Animal Research Center, ARS, USDA, Clay Center, NE 68933, and
‡Department of Animal and Poultry Sciences,
Virginia Polytechnic Institute and State University, Blacksburg 24061

ABSTRACT: Ovulation rate, serum hormone concentrations, follicular fluid (FFL) concentrations of steroids and IGF, IGF binding protein (IGFBP) activity in FFL, and follicular IGF-I and -II mRNA were compared during the follicular phase among five genotypes of ewes: Finn (F), Composite III (C), 1/2 Booroola Merino (B) × 1/2 F (B×F), 1/2 F × 1/2 C (F×C), 1/2 B × 1/2 C (B×C). Composite III ewes were a Columbia × Suffolk × Hampshire crossbred. Ovulation rates for F (n = 7), C (n = 5), B×F (n = 6), F×C (n = 3), and B×C (n = 8) ewes were 3.1, 1.6, 3.8, 2.9, and 2.9 (Pooled SEM = .5), respectively. Concentrations of IGF-I in FFL were 53% greater ($P < .05$) in large (≥ 4.1 mm) than in small (< 4.1 mm) follicles but did not differ ($P > .10$) among genotypes. In contrast, FFL IGF-II concentrations were greater ($P < .05$) in B×C and B×F ewes than in C or F×C ewes but did not differ between small and large follicles. Ligand blotting revealed that IGFBP activity of three species (34, 27 to 29, and 24 kDa) were lower ($P < .05$) in FFL of large than in FFL of small follicles but did not differ ($P > .10$) among genotypes. Follicular wall IGF-I mRNA and IGF-II mRNA was detected in 5 and 32% of the samples from preovulatory follicles, respectively, using reverse transcriptase-PCR and ethidium-bromide staining. Ovarian IGF-I mRNA levels, assessed by Northern analysis, in B×F and B×C ewes were greater ($P < .05$) than those in C ewes; ovarian IGF-I mRNA levels in F and F×C ewes were intermediate and did not differ ($P > .10$) from those in C ewes. Small follicles from B×C and B×F ewes had severalfold greater ($P < .05$) estradiol concentrations than those from F or C ewes, whereas large follicles from B×F ewes had twice ($P < .05$) the estradiol concentrations of follicles from F or C ewes. Progesterone in FFL did not differ among genotypes. Serum LH, FSH, inhibin, IGF-I, and progesterone did not differ ($P > .10$) among genotypes. In conclusion, higher ovulation rates in B-cross ewes (i.e., B×F and B×C vs C) were associated with greater levels of FFL IGF-II, FFL estradiol, and ovarian IGF-I mRNA but were not associated with differences in FFL progesterone, IGF-I, or IGFBP.

Key Words: Insulin-Like Growth Factor, Ovulation Rate, Follicular Fluid, Ovaries, Sheep, Binding Proteins

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Introduction

The IGF have been postulated to serve as intraovarian regulators of follicular growth and differentiation in mammals (Geisthovel et al., 1990; Hammond et al., 1991; Giudice, 1992). Specifically, in vitro studies have established that ovarian granulosa cells from porcine and bovine follicles can secrete IGF-I (Hammond et al., 1991; Spicer et al., 1993a) and that IGF-I and(or) IGF-II stimulate mitogenesis and steroidogenesis in porcine (Baranao and Hammond, 1984), ovine (Monniaux and Pisselet, 1992), and bovine (Spicer et al., 1993a) granulosa cells. In pigs and cattle, ovarian tissues contain mRNA for IGF (Spicer

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[2]To whom correspondence should be addressed.

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et al., 1993a; Samaras et al., 1994), and IGF-I concentrations in follicular fluid (**FFL**) increase with follicular size (Spicer et al., 1988, 1992; Echternkamp et al., 1990). However, no evidence exists to support the idea that intraovarian concentrations of IGF-I or IGF-II are associated with follicular growth and differentiation in sheep.

Because concentrations of IGF-I in peripheral blood seem to be genetically determined in several mammals (Blair et al., 1988; Jones et al., 1991; Spicer et al., 1992), and may mediate a genetic component of multiple ovulations in cattle (Echternkamp et al., 1990), we investigated the possibility that various genotypes of sheep with increased ovulation rates may have increased concentrations of IGF-I or -II in FFL and(or) increased amounts of ovarian IGF-I or -II mRNA. To this end, we measured concentrations of IGF-I, IGF-II, progesterone, and estradiol in FFL, IGF binding protein activity in FFL, levels of IGF-I mRNA in ovarian tissue, and IGF-I and IGF-II mRNA in follicular walls collected simultaneously during the preovulatory period from ewes of different genotypes exhibiting low, intermediate, and high ovulation rates. In addition, serum concentrations of LH, FSH, inhibin, progesterone, and IGF-I were measured during the preovulatory period.

Materials and Methods

Animals and Sample Collection. Ten ewes each from five genotypes were initially used: Finn (**F**), Composite III (**C**; 1/2 Columbia, 1/4 Suffolk, 1/4 Hampshire), 1/2 Booroola Merino (**B**) × 1/2 **F** (B×F), 1/2 F × 1/2 C (F×C), and 1/2 B × 1/2 C (B×C). Booroola sires were homozygous for the fecundity gene. All ewes were maintained in a common flock at the Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska and had ad libitum access to a medium-quality grass hay. Ewes were approximately 5 yr of age during the study and averaged 66, 92, 57, 76, and 67 ± 2 kg BW for F, C, B×F, F×C, and B×C genotypes.

Estrous cycles of the ewes were synchronized with two injections (i.m.) of 15 mg of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse™, Upjohn, Kalamazoo, MI) 9 d apart in October 1990, and ovaries of ewes were obtained via midventral ovariectomy 48 h after the second $PGF_{2\alpha}$ injection. All ewes undergoing ovariectomies were given xylazene (Rompum™, 1 mg per 4.54 kg body weight i.m.) plus a local anesthetic (2% lidocaine) before surgery. Starting 6 h before the second $PGF_{2\alpha}$ injection, blood samples were collected via jugular venipuncture every 6 h until 42 h after the second $PGF_{2\alpha}$ injection. Serum was harvested and frozen at −20°C until it was analyzed for LH, FSH, inhibin, progesterone, and IGF-I. Immediately following ovariectomy, the numbers of follicles 1 to 4.0 mm (small) and ≥ 4.1 mm (large) on the ovarian surface were recorded. This size classification was based on previous studies indicating that most ovine ovulatory follicles were > 4 mm (Webb and England, 1982) and that ovine follicles > 4 mm produced greater amounts of estradiol in vitro than follicles < 4 mm (Mann et al., 1992). Follicular fluid (FFL) from 1- to 4.0-mm follicles was collected and combined within each ovary, whereas FFL from follicles ≥ 4.1 mm was collected individually. Follicular walls (granulosa cells plus theca interna) of all follicles ≥ 4.1 mm were dissected from each pair of ovaries, collected individually or pooled within ewe, placed in cryovials, frozen in liquid nitrogen and stored at −70°C for subsequent mRNA analysis. The remaining residual ovary was also frozen in liquid nitrogen and stored at −70°C for subsequent mRNA analysis. During follicle dissection, ovaries were placed on ice; the interval from ovariectomy to freezing of the last sample from each ewe averaged 30 ± 2 min.

Radioimmunoassays. Concentrations of IGF-I, progesterone, LH, FSH, and inhibin in serum were quantified by previously validated RIA (Echternkamp and Lunstra, 1984; Schanbacher, 1988; Spicer et al., 1993b). Sensitivity of the IGF-I RIA was 18 pg/tube, and the inter- and intraassay CV were 12.5 and 9.4%, respectively. Sensitivity of the progesterone RIA was 20 pg/mL, and the inter- and intraassay CV were 11.2 and 9.6%, respectively. Serum samples were analyzed for LH, FSH, and inhibin in a single RIA for each hormone. The intraassay CV for the LH, FSH, and inhibin RIA were 7.4, 7.8, and 14.4%, respectively. Concentrations of IGF-I, progesterone, and estradiol in FFL were quantified by previously validated RIA (Echternkamp et al., 1990; Spicer and Enright, 1991). The inter- and intraassay CV were 16 and 9, 26 and 15, and 17 and 14%, respectively, for IGF-I, progesterone, and estradiol RIA using a pool of FFL that averaged 55 ± 2, 30 ± 2, and 20 ± 1 ng/mL, respectively.

Concentrations of IGF-II in FFL were quantified by the method of Bowsher et al. (1991) using a formic acid-acetone extraction, except that recombinant bovine IGF-II was used as the tracer and standard. This procedure resulted in parallelism between the bovine IGF-II standard (Monsanto, St. Louis, MO) and ovine serum using a rat IGF-II antiserum (Amono International Enzyme Co., Troy, VA). Cross-reactivity of IGF-I in the IGF-II RIA was less than 5.0%. The intraassay CV was 6.8%.

Insulin-Like Growth Factor I Binding Protein Activity and Ligand Blots. Total IGF-I binding protein (**IGFBP**) activity in FFL was determined by incubation of FFL with [^{125}I]IGF-I as previously described (Moses et al., 1979; Spicer et al., 1992). Total IGFBP activity in FFL was measured in samples pooled within each ewe by follicle size (small vs large) to provide two IGFBP activity determinations per ewe. Intra- and interassay CV were 5.2 and 9.2%, respectively.

To evaluate the various molecular weight species of the IGFBP, one-dimensional SDS-PAGE, under non-reducing conditions, was performed as described previously (Laemmli, 1970; Howard and Ford, 1992). Proteins were separated on a 12% polyacrylamide separating gel and a 4% stacking gel, with 1 μL of follicular fluid plus 24 μL of buffer loaded per lane. After electrophoresis of samples, proteins were electrophoretically transferred to nitrocellulose, and ligand was blotted for IGFBP activity with use of [^{125}I]IGF-I (Hossenlopp et al., 1986). Band intensity on autoradiographs was characterized by scanning densitometry.

Isolation and Quantification of Insulin-Like Growth Factor I mRNA. Total RNA was extracted from left and right ovaries (excluding walls of follicles ≥ 4.1 mm) using the modified acid guanidinium isothiocyanate extraction procedure of Puissant and Houdebine (1990). Ten milligrams of total RNA was loaded per lane and separated by electrophoresis in 1% agarose-formaldehyde gels. The RNA was transferred to Nitroplus™ membranes (Micron Separations, Westborough, MA) by capillary action and fixed to the membrane by UV crosslinking. Blots were hybridized with a ^{32}P-labeled oligonucleotide specific for ovine IGF-I (oIGF-I-3a 5′ GTCTCCGCACACGAACTGGA 3′, exon 3, nt 306–325; Ohlsen et al., 1993) and washed following the manufacturer's protocol. Blots were exposed to Kodak XAR-5 autoradiographic film (Eastman Kodak, Rochester, NY) at −80°C for 7 d with intensifying screens. The size of each of the oIGF-I transcripts was estimated using RNA markers (.24 to 9.5 kilobases; GIBCO-BRL, Gaithersburg, MD). Ovine IGF-I mRNA was quantified by densitometry and normalized to the intensity of the large ribosomal RNA to correct for unequal RNA loading. The quantities of ovine IGF-I mRNA are expressed as arbitrary densitometric units.

Detection of Insulin-Like Growth Factor-I and -II mRNA in Follicular Walls Using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from individual or pooled follicular wall samples using the guanidinium isothiocyanate procedure described in the previous section. The presence of IGF-I and IGF-II mRNA was assayed by RT-PCR according to the procedure of Ohlsen et al. (1994). Samples (n = 107) were randomly assigned to one of nine groups and run in the RT-PCR reaction. Briefly, IGF-I and IGF-II cDNA were synthesized simultaneously from .3 μg of total RNA using MMLV reverse transcriptase and oligonucleotide primers oIGF-I-6a (5′ CTGCACTCCCTCTGCTTGTG 3′, exon 6, nt 121–140) and oIGF-II-10b (5′ TCAGCGGACGGTGACTCTTG 3′, exon 10, nt 219–238). An aliquot of the cDNA reaction was then used for separate IGF-I and IGF-II PCR reactions. For IGF-I, oligonucleotide primers oIGF-I-4a (5′ GAGCCTTGGGCATGTCGGTG 3′, exon 4, nt 219–238) and oIGF-I 3c (5′ GATGCCAGTCACATCCTCCTCGC 3′, exon 3, nt 190–212) were used. For IGF-II, oligonucleotide primers oIGF-II-10c (5′ TGAGCGCCTCCAGCTCCTTG 3′, exon 10, nt 192–211) and oIGF-II-9a (5′ GGCATCGTGGAAGAGTGT 3′, exon 9, nt 278–295) were used. Nucleotide numbering is from Ohlsen et al. (1993) for IGF-I and Ohlsen et al. (1994) for IGF-II. For each PCR run, negative and positive control samples were included that used all reagents except that water or 100 ng of IGF-I or IGF-II cDNA plasmid was substituted for the reverse transcriptase product. Products of the PCR reactions for IGF-I (329 bp) and IGF-II (256 bp) were separated on 1% agarose gels with a 123-bp ladder (GIBCO-BRL) as size markers. The gels were stained with ethidium bromide and photographed under UV light. Each sample was assigned as positive (containing the expected PCR product) or negative for IGF-I and IGF-II. The PCR bands were confirmed to be IGF-I or IGF-II by Southern blot analysis using ovine IGF-I or IGF-II cDNA as probes (data not shown).

Statistical Analyses. Serum hormone data were subjected to the GLM procedures of SAS (1988) using a split-plot ANOVA with genotype as the main plot and ewes nested within genotype as its error term; hour before ovariectomy was the subplot with interactions. Serum IGFBP data were analyzed as a one-way ANOVA and specific serum means were compared with FFL IGFBP means by Student's *t*-test. Follicular fluid hormone data for small and large follicles were subjected to ANOVA with genotype and follicle size as main effects plus interactions. Mean comparisons were assessed using Fisher's protected least significant difference procedure unless stated otherwise. The CONTRAST statement for GLM procedures of SAS (1988) was used to evaluate potential differences in variables due to ovulation rate and heterosis using four predetermined contrasts: C vs B×F for low vs high ovulation rate; C vs F×C plus F plus B×C for low vs medium ovulation rate; B×F vs F×C plus F plus B×C for high vs medium ovulation rate; and F×C vs F plus C for a heterosis effect. Only data from ewes that were in the follicular phase of the estrous cycle were included in the analyses; some did not respond (i.e., presence of morphologically regressing corpora lutea and serum progesterone < 2.0 ng/mL for three consecutive samples) to the two injections of $PGF_{2\alpha}$ whereas others ovulated before ovariectomy; seven, three, five, four, and two ewes in F×C, F, C, B×F, and B×C groups, respectively, were not in the follicular phase of the estrous cycle. Relationships among selected variables were evaluated with regression and simple correlation analyses (Pearson correlation coefficients; SAS, 1988). The percentage of follicular wall samples containing either IGF-I or IGF-II mRNA for each genotype was analyzed by logistic regression using the CATMOD procedure of SAS (1988).

Table 1. Average ovarian weight (OVWT), number of small (1 to 4 mm) and large (≥ 4.1 mm) follicles, average diameter of large follicles, and ovulation rate (OR) of F×C, F, C, B×F, and B×C ewes 48 hours after prostaglandin-$F_{2\alpha}$ synchronization

Genotype	No. of ewes in the follicular phase	OR	No. of small follicles	No. of large follicles	Avg diameter, mm	OVWT, g
F×C	3	2.9^{a}	10.3	2.3	6.2^{a}	1.6^{a}
F	7	3.1^{a}	14.0	3.1	5.6^{bc}	1.5^{a}
C	5	1.6^{b}	12.0	2.4	5.7^{ab}	2.4^{b}
B×F	6	3.8^{c}	8.8	1.8	5.0^{d}	1.5^{a}
B×C	8	2.9^{a}	10.8	2.1	5.2^{cd}	1.5^{a}
SEM	—	.5	1.7	.4	.2	.1

^{a,b,c,d}Means without a common superscript differ ($P < .05$).

Results

Numbers and Size of Follicles. Ovulation rate (assessed by the number of corpora albicantia present at ovariectomy) was greatest in B×F ewes and lowest in C ewes (Table 1). Ovulation rate in F×C, F, and B×C ewes did not differ ($P > .10$) (Table 1). The number of small (1.0 to 4.0 mm) and large (≥ 4.1 mm) follicles averaged 11.3 and 2.4 per ovary, respectively, and did not differ among genotypes (Table 1). Average diameter of the large follicles for F×C and C ewes was greater than for B×F and B×C ewes ($P < .05$) (Table 1). Diameter of large follicles in F ewes was intermediate (Table 1).

Ovarian weight was greater ($P < .05$) for C ewes than for F×C, F, B×F, or B×C ewes (Table 1).

Concentrations of Luteinizing Hormone, Follicle-Stimulating Hormone, Inhibin, Progesterone, and Insulin-Like Growth Factor I in Serum. Because concentrations of LH, FSH, inhibin, progesterone, and IGF-I in serum did not differ ($P > .10$) among genotypes and there was no genotype × time interaction, hormone values for all ewes defined as preovulatory (n = 29) were combined across genotype and presented in Figure 1 (except for progesterone; see below). All five hormones changed ($P < .05$) with time after $PGF_{2\alpha}$. None of the 29 ewes exhibited a LH surge before 36 h after $PGF_{2\alpha}$; concentrations of LH increased only at 42 h after $PGF_{2\alpha}$. Concentrations of IGF-I increased ($P < .05$) within 18 h post-$PGF_{2\alpha}$, whereas FSH concentrations decreased ($P < .05$) within 18 h after $PGF_{2\alpha}$. Inhibin decreased ($P < .05$) between 0 and 6 h and then increased ($P < .05$) by 42 h (Figure 1). Progesterone was measured in alternate samples and decreased ($P < .05$) by 12 h after $PGF_{2\alpha}$ (0 h = 4.9 ng/mL; 12 h = 2.0 ng/mL; 24 h = .7 ng/mL; 36 h = .7 ng/mL).

Concentrations of Insulin-Like Growth Factor-I, -II, Estradiol and Progesterone in Follicular Fluid. Concentrations of IGF-I in FFL did not differ ($P > .10$) among genotypes (Table 2) but were 53% greater ($P < .05$) in large than in small follicles (Figure 2). Concentrations of IGF-I averaged across large follicles within left and right ovaries were correlated ($P < .05$) with serum IGF-I (averaged over −6 to 42 h after $PGF_{2\alpha}$) (r = .39 and .52 for left and right ovaries, respectively); IGF-I concentrations in small follicles were not correlated significantly with serum IGF-I.

Concentrations of IGF-II in FFL varied ($P < .05$) with genotype (Figure 3) but did not differ ($P > .10$) between small (508 ± 40 ng/mL) and large (562 ± 26 ng/mL) follicles. Combined across small and large follicles, IGF-II concentrations were lowest in C ewes, intermediate in F, F×C, and B×F ewes, and greatest in B×C ewes (Figure 3). Specific predetermined contrasts between various genotypes revealed that C ewes had lower ($P < .01$) FFL IGF-II concentrations than B×F ewes or F×C, F, and B×C ewes; FFL IGF-II in B×F ewes did not differ from that in F×C, F, and B×C ewes (Table 2).

Concentrations of estradiol varied ($P < .01$) with genotype and follicle size (Figure 4). Estradiol concentrations in large follicles were greater than in small follicles (66 vs 25, SEM = 8 ng/mL). Small follicles from B×C and B×F ewes had severalfold greater ($P < .05$) FFL concentrations of estradiol than follicles from F or C ewes. Large follicles from B×F ewes had greater ($P < .05$) FFL concentrations of estradiol than follicles from F or C ewes (Figure 4). Specific predetermined contrasts between various genotypes revealed that B×F ewes had higher ($P < .01$) FFL estradiol concentrations than C ewes, and F×C, F, and B×C ewes; FFL estradiol concentrations did not differ ($P > .10$) between C ewes and F×C, F, and B×C ewes (Table 2).

Concentrations of progesterone in FFL of small and large follicles did not differ ($P > .10$) among genotypes (Table 2). However, progesterone concentrations in large follicles (47 ± 5 ng/mL) were greater ($P < .10$) than in small follicles (28 ± 8 ng/mL).

Total Insulin-Like Growth Factor Binding Protein Activity in Follicular Fluid. Total IGFBP activity in FFL did not differ ($P > .10$) among genotypes but was

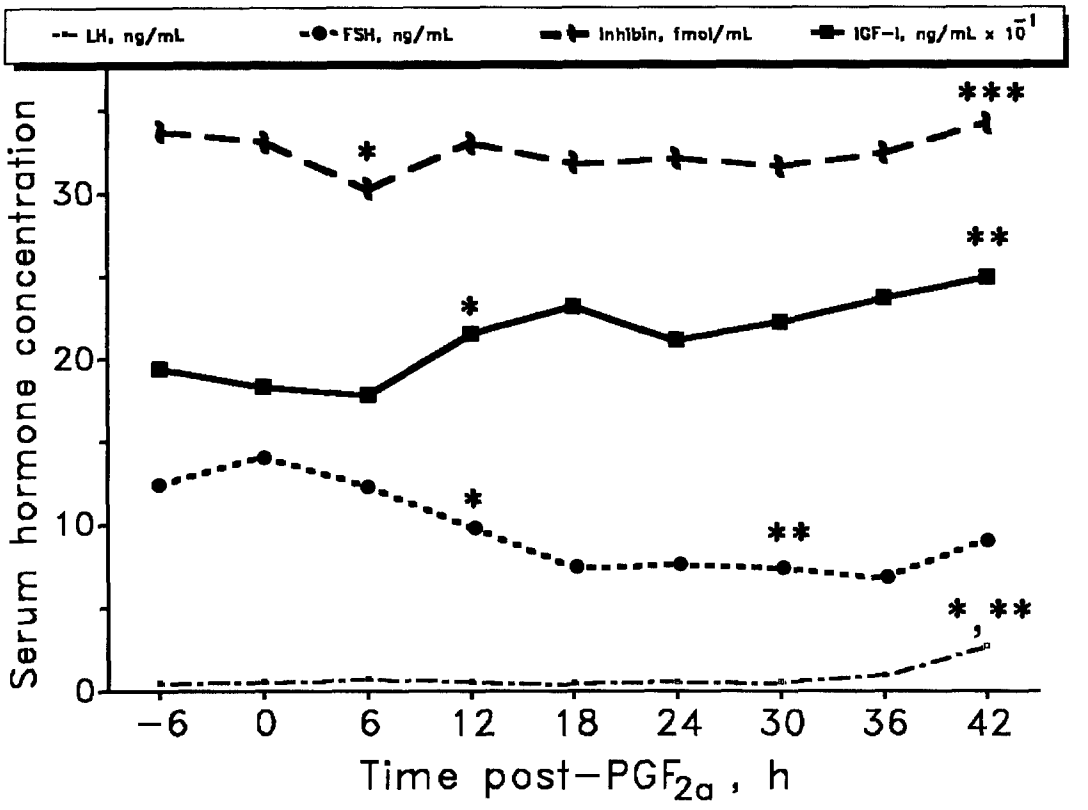

Figure 1. Concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), inhibin, and insulin-like growth factor I (IGF-I) from −6 h to 42 h after prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$). Data from F×C, F, C, B×F, and B×C ewes were pooled. *First value that differs ($P < .05$) from 0-h value; **first value that differs ($P < .05$) from 12-h value; ***differs ($P < .05$) from 6-h value. Pooled SEM = .5 ng/mL, .8 ng/mL, 1.1 fmol/mL and 8.3 ng/mL for LH, FSH, inhibin, and IGF-I, respectively.

greater ($P < .01$) in small follicles than in large follicles (9.4% vs 3.9 ± 1.0% [^{125}I]IGF-I bound per 10 μL, respectively).

Various Forms of Insulin-Like Growth Factor Binding Protein Activity in Follicular Fluid and Serum. Ligand blots revealed that at least five forms of IGFBP activity existed in sheep FFL and serum (Figure 5). Because of the variation in migration of IGFBP observed between 27 and 29 kDa, all bands in this molecular weight range were combined for analysis. In addition, the two major IGFBP between the 40- and 44-kDa range were combined and identified as IGFBP-3. Two other distinct bands were observed with estimated molecular weights of 24 and 34 kDa (IGFBP-2). Genotype had no effect ($P > .10$) on the amount of the five forms of IGFBP in FFL or serum (data not shown). However, follicle size affected ($P < .05$) the amount of binding activity of

Table 2. Summary of specific mean contrasts between various genotypes of ewes in an attempt to determine effect of ovulation rate (OR) and heterosis on selected follicular fluid (FFL) variables

Contrast	Comparison	FFL variable[a]			
		IGF-I	IGF-II	E2	PROG
		$P <$			
C vs B×F	Low vs high OR	.72	.01	.01	.35
C vs F×C + F + B×C	Low vs medium OR	.71	.01	.24	.40
B×F vs F×C + F + B×C	High vs medium OR	.96	.98	.01	.79
F×C vs F + C	Heterosis	.72	.71	.29	.42

[a]E2 = estradiol; PROG = progesterone.

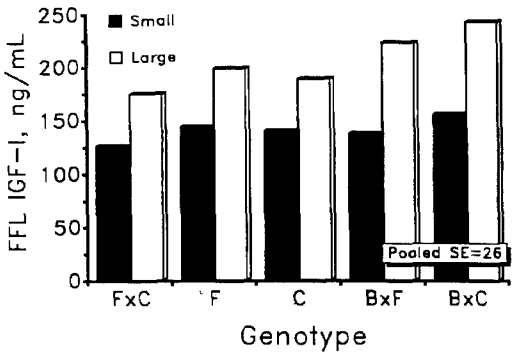

Figure 2. Least squares means of concentrations of insulin-like growth factor I (IGF-I) in follicular fluid (FFL) collected from small (1 to 4 mm) and large (4.1 to 8.0 mm) follicles of F×C, F, C, B×F, and B×C ewes. No genotype effect ($P > .10$) was observed; however, an effect ($P < .05$) of follicle size was observed.

IGFBP-2, 27 to 29 kDa, and 24 kDa IGFBP (Figure 6) such that small follicles had severalfold greater ($P < .05$) levels of these forms of IGFBP than did large follicles. Levels of binding activity of IGFBP-3 in FFL did not differ between large and small follicles (Figure 6). Total intensity (sum of individual bands) of all IGFBP forms was also greater ($P < .05$) in small than in large follicles (24.61 vs 16.85 relative area units). The IGFBP-3 (40 to 44 kDa) binding activity was greater ($P < .05$) in serum than in FFL of small and large follicles (Figure 6).

Amounts of Insulin-like Growth Factor-I mRNA in Ovarian Tissue. Northern blot analysis revealed a predominant 7.5-kilobase transcript in whole ovaries (Figure 7). Ovarian IGF-I mRNA levels were greater

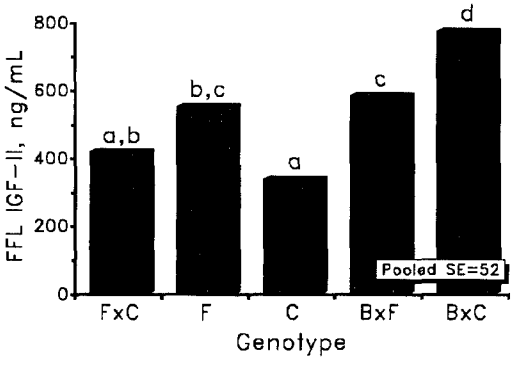

Figure 3. Least squares means of concentrations of insulin-like growth factor II (IGF-II) in follicular fluid (FFL) collected from small (1 to 4 mm) and large (4.1 to 8.0 mm) follicles of F×C, F, C, B×F, and B×C ewes. No size effect ($P > .10$) was observed, and thus data were pooled across follicle sizes. However, an effect ($P < .05$) of genotype was observed. ^{a,b,c,d}Means without a common letter differ ($P < .05$).

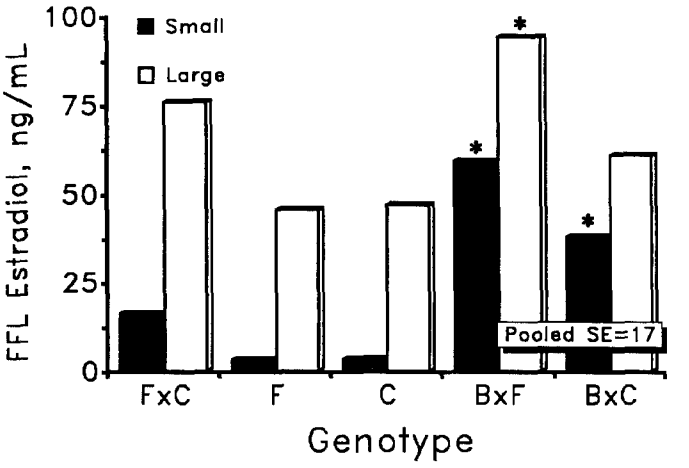

Figure 4. Least squares means of concentrations of estradiol in follicular fluid (FFL) collected from small (1 to 4 mm) and large (4.1 to 8.0 mm) follicles of F×C, F, C, B×F, and B×C ewes. Effects ($P < .05$) of genotype and follicle size were observed. *$P < .05$ vs F and C means within follicle size group.

($P < .05$) in B×F and B×C ewes than in C ewes; ovarian IGF-I mRNA levels in F and F×C ewes were intermediate and did not differ ($P > .10$) from those in C ewes (Figure 8).

Presence of Insulin-Like Growth Factor-I and -II mRNA in Follicular Wall Samples. Because IGF-I mRNA in follicular walls was undetectable by Northern blot, a more sensitive RT-PCR assay was used. Figure 9 shows representative gels of the RT-PCR analysis for IGF-I and IGF-II mRNA from some of the follicular wall samples analyzed. In Panel A, an IGF-I PCR product of 329 bp (arrow) was present in lanes 2 and 12, whereas in Panel B, an IGF-II PCR product of 256 bp (arrow) was present in lanes 3, 6, 7, 9, and 11. Qualitative assessment of ethidium bromide-stained gels revealed that 5 to 8% (4 of 75 samples from preovulatory ewes; 9 of 107 samples from all ewes) of the follicular wall samples contained IGF-I mRNA, whereas 32 to 34% (24 of 75 samples from preovulatory ewes; 36 of 107 samples from all ewes) of the follicular wall samples contained IGF-II mRNA. There were no apparent differences among genotypes in the frequency of occurrence of IGF-I or -II mRNA (data not shown). A chi-square test of independence for the presence or absence of mRNA in follicle wall samples (based on ethidium bromide-stained gels) revealed that a much higher ($P < .01$) proportion of follicular wall samples contained IGF-II mRNA than IGF-I mRNA. The 5 to 34% is likely an underestimation of the percentage of follicular walls that contain IGF-I or IGF-II mRNA, because numerous samples lacking a detectable PCR product by ethidium-bromide staining revealed a hybridizing band when determined by Southern analysis. Specifically, when 2.5- to

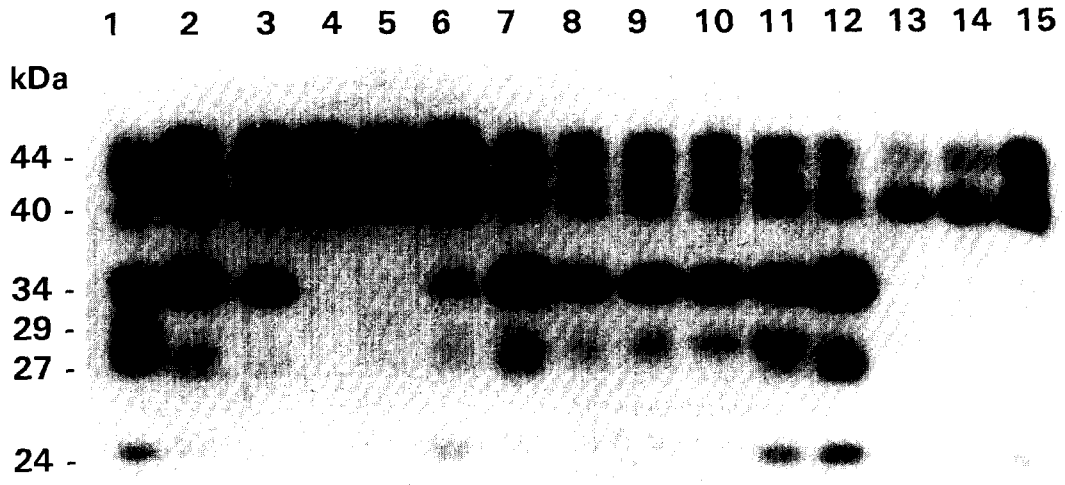

Figure 5. A representative ligand blot analysis of insulin-like growth factor binding proteins in serum and follicular fluid (FFL) of ewes ovariectomized 48 h after prostaglandin-$F_{2\alpha}$ synchronization. Follicular fluid samples (1 μL) from three individual ewes were analyzed as described in Materials and Methods. Lane 1: pool of bovine FFL; lanes 2 to 5: FFL samples from large follicles of a C ewe; lane 6: serum of the same C ewe in lanes 2 to 5; lane 7: FFL pool from small follicles of a F ewe; lanes 8 to 10: FFL samples from large follicles of the same F ewe in lane 7; lane 11: serum of the same F ewe in lanes 7 to 11; lane 12: FFL pool from small follicles of a F×C ewe; lanes 13 to 14: FFL samples from large follicles of the same F×C ewe in lane 12; lane 15: serum of the same F×C ewe in lanes 12 to 14.

3.0- times the background was used as a limit of detectability on Southern analysis, 26% and 41% of the follicular walls (all samples) contained IGF-I and IGF-II mRNA, respectively.

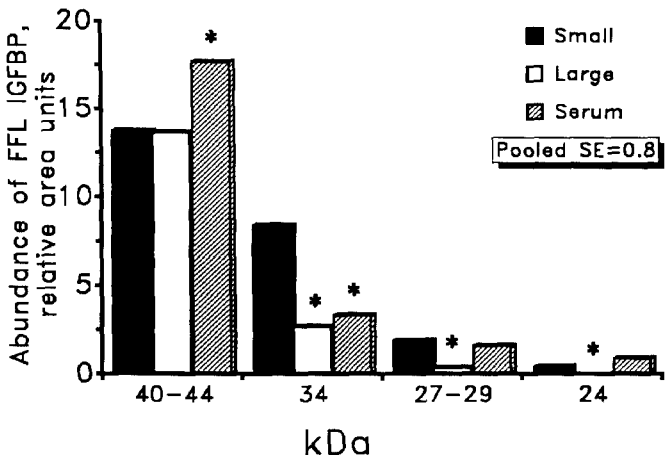

Figure 6. Least squares means of relative abundance of insulin-like growth factor binding protein (IGFBP) forms in follicular fluid (FFL) collected from small (1 to 4 mm) and large (4.1 to 8.0 mm) follicles and in serum of F×C, F, C, B×F, and B×C ewes. No genotype effect ($P > .10$) was observed for FFL or serum; however, an effect ($P < .05$) of follicle size was observed for FFL. *$P < .05$ vs small follicle mean as assessed by Student's t-test.

Discussion

The genetic determinant for ovulation rate in sheep differs among breeds and(or) strains (Hanrahan, 1991; Montgomery et al., 1992). However, the physiologic mechanism mediating differences in ovulation rate among most genotypes of ewes is unknown and cannot be readily explained by systemic hormonal changes (e.g., FSH as shown for Booroola ewes; Montgomery et al., 1992). Because IGF-I has been implicated as a potential regulator of twinning in cattle (Echternkamp et al., 1990), we hypothesized that IGF-I may be one of these factors regulating ovulation rate in sheep. However, concentrations of IGF-I in serum and FFL did not differ among the various genotypes of sheep that ranged in ovulation rate from 1.6 to 3.8 in the present study. Similarly, our previous studies in sheep have revealed no clear-cut relationship between ovulation rate and concentrations of IGF-I in blood (Spicer and Zavy, 1992; Spicer et al., 1993b). In fact, Finn ewes selected for high ovulation rate (ovulation rate = 3.3) had 20% lower plasma IGF-I concentrations throughout the estrous cycle than did Finn ewes selected for low ovulation rate (ovulation rate = 1.3) (Spicer et al., 1993b). Thus, it seems that increased systemic and FFL IGF-I do not account for increased ovulation rate in some genotypes of ewes.

Assessment of morphological and functional characteristics of antral follicles from low- and high-prolificacy ewes indicates that presumptive preovulatory follicles from prolific ewes are smaller and

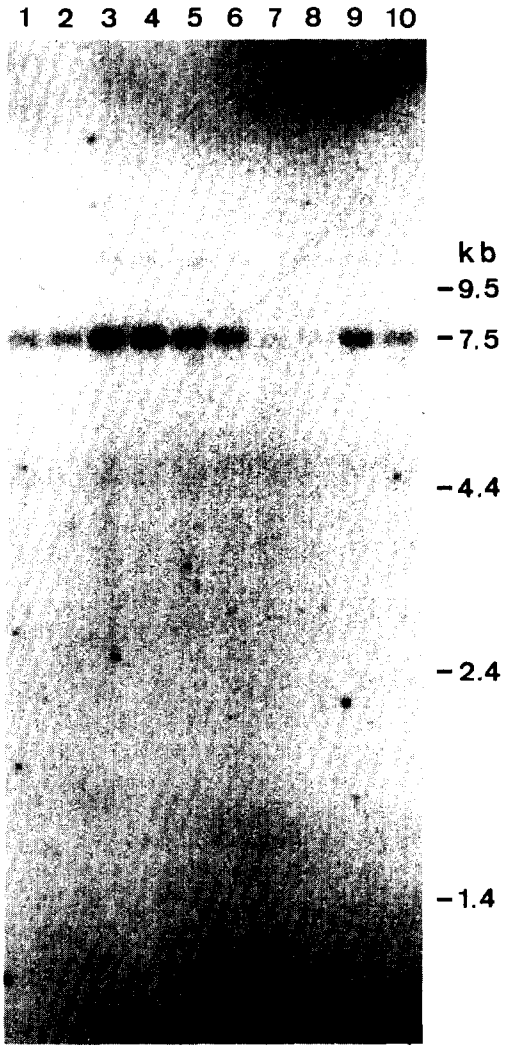

Figure 7. A representative Northern blot of ovarian insulin-like growth factor I (IGF-I) messenger ribonucleic acid (mRNA) in ewes ovariectomized 48 h after prostaglandin-$F_{2\alpha}$ synchronization. RNA samples from representative sets of ovine ovaries are shown in lanes 1 to 10. Left and right ovaries from five individual ewes are shown as pairwise combinations with odd- and even-numbered lanes containing RNA from the left and right ovaries, respectively. Lanes 1 and 2: ovarian samples from a B×C ewe (#098); lanes 3 and 4: ovarian samples from a B×C ewe (#101); lanes 5 and 6: ovarian samples from a B×C ewe (#108); lanes 7 and 8: ovarian samples from a F ewe (#285); lanes 9 and 10: ovarian samples from a F ewe (#458).

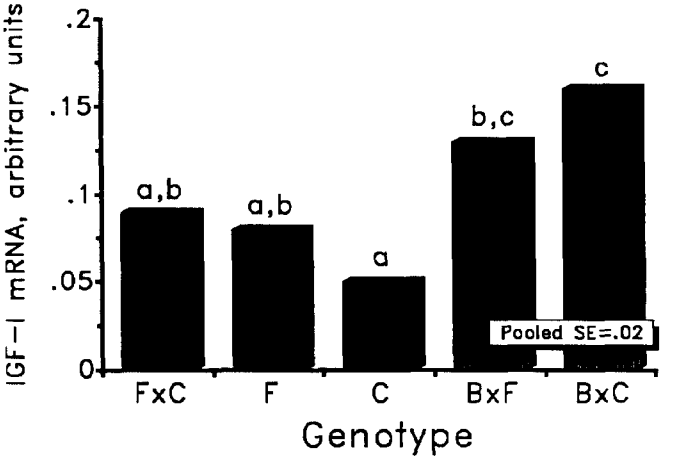

Figure 8. Relative expression of ovarian insulin-like growth factor I (IGF-I) messenger ribonucleic acid (mRNA) in F×C, F, C, B×F, and B×C ewes. ^{a,b,c}Means without a common letter differ ($P < .05$).

contain fewer granulosa cells but produce more estrogen per granulosa cell and thus contain greater estradiol concentrations in FFL than those from less-prolific ewes (McNatty et al., 1986; Webb et al., 1989; Driancourt et al., 1990). Similarly, we observed greater estradiol concentrations in FFL of small and large follicles from prolific B-cross ewes (i.e., B×F and B×C genotypes) than that from the low-prolificacy ewes (i.e., C genotype). In view of the fact that the average diameter of large follicles of B-cross ewes were smaller than F×C and C ewes, it is likely that some of these small follicles with greater estradiol concentrations in B-cross ewes of the present study were preovulatory follicles. In comparison, progesterone concentrations in FFL did not differ among low- and high-prolificacy genotypes in the present or a previous study (Driancourt and Hanrahan, 1991). The hormonal mechanism whereby follicles from high-prolificacy ewes specifically produce more estrogen per follicle remains unknown.

The present study provides the first evidence that the ovine ovary is capable of producing IGF-I and IGF-II. Previous reports indicated that bovine granulosa cells produce IGF-I in vitro and contain IGF-I mRNA (Spicer et al., 1993a). Human granulosa cells do not produce IGF-I or contain hormonally inducible IGF-I mRNA (Voutilainen and Miller, 1987; Geisthovel et al., 1989; Ramasharma and Li, 1989) but instead contain hormonally inducible IGF-II mRNA (Voultilainen and Miller, 1987). In the present study, IGF-I mRNA was detectable in ovine follicular walls (theca interna plus membrana granulosa) by Northern blot analysis. However, the more sensitive Southern blot analysis revealed a significant proportion of follicular wall samples to be positive for IGF-I and IGF-II mRNA (26 and 41%, respectively). It is presumed that follicular walls that were negative for the presence of mRNA using ethidium-bromide staining but positive for mRNA using Southern blot analysis contained significantly less mRNA than those positive for both procedures. More of the ovine follicles contained greater levels of IGF-II than of IGF-I mRNA, so these results imply that granulosa and(or)

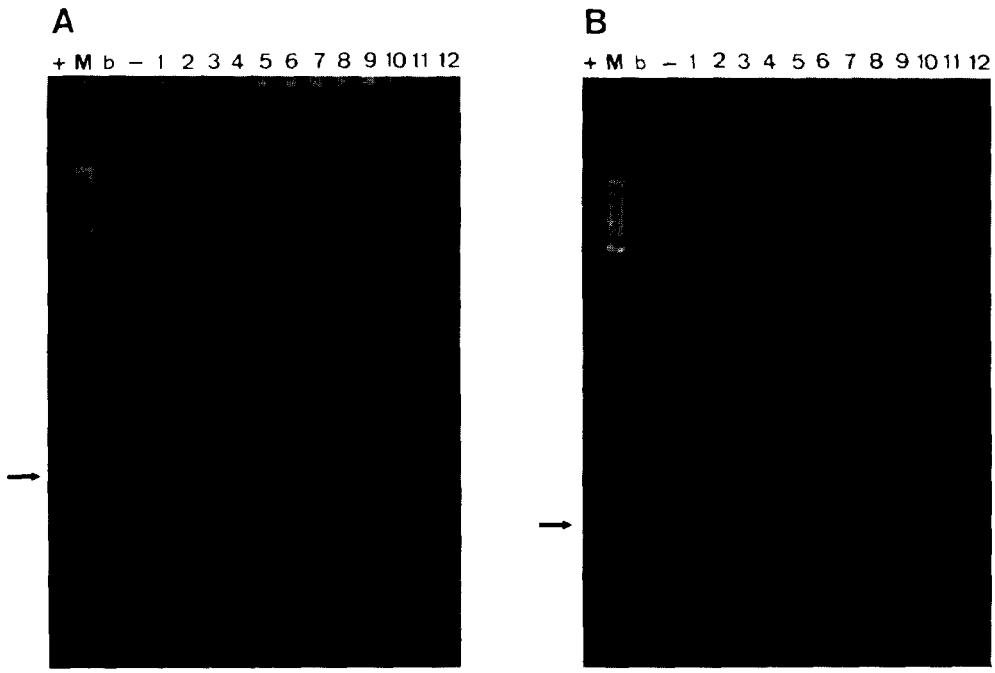

Figure 9. Detection of insulin-like growth factor I (IGF-I; Panel A) and IGF-II (Panel B) messenger ribonucleic acid (mRNA) by reverse transcriptase-polymerase chain reaction (RT-PCR) on the ethidium bromide stained agarose gel. Samples of total RNA (.3 μg) from ovine follicular walls were reverse-transcribed and amplified with oligonucleotide primers as described in Materials and Methods. Representative follicular wall samples from eight ewes are shown in Panel A (lanes 1 to 12) and from nine ewes in Panel B (lanes 1 to 12). Lanes (+) and (–) were positive and negative PCR controls, respectively, containing cDNA plasmid or water substituted for the product of the reverse transcriptase reaction. Lane b was left blank and lane M contained a 123-bp ladder as size markers. Panel A: lanes 1 to 3, individual follicular wall samples from a F ewe; lane 4, a sample from a F×C ewe; lane 5, a sample from a B×F ewe; lane 6, a sample from a C ewe; lanes 7 to 8, samples from a B×C ewe; lane 9, a sample from another B×C ewe; lanes 10 to 11, samples from another F ewe; lane 12, a pool of two samples from a B×F ewe. Panel B: lane 1, individual follicular wall sample from a F×C ewe; lanes 2 to 3, samples from a B×F ewe; lane 4, a sample from a C ewe; lane 5, a sample from a F ewe; lanes 6 to 7, samples from another F ewe; lane 8, a sample from another B×F ewe; lanes 9 to 10, samples from another F ewe; lane 11, a sample from a B×C ewe; lane 12, a sample from another B×F ewe.

thecal cells in ovine follicles produce more IGF-II than IGF-I and are less likely to be significant source(s) of IGF-I than of IGF-II. In support of this suggestion, we observed that FFL concentrations of IGF-II were twofold higher than FFL IGF-I. In addition to localized intraovarian IGF-I gene expression, current evidence suggests that a portion of the IGF-I in FFL is derived from blood serum and, thus, accounts for the positive correlation between serum and FFL IGF-I concentrations in the present study and previous studies in cattle (Echternkamp et al. 1990) and swine (Spicer et al., 1992; Echternkamp et al., 1994b).

Although FFL IGF-I levels and follicular wall samples containing IGF-I mRNA did not differ among genotypes, ovarian levels of IGF-I mRNA were higher in B-cross ewes (ovulation rate = 3 to 4) than in C ewes (ovulation rate = 1.6). The B×C and F×C ewes had similar ovulation rates, but B×C ewes had significantly greater ovarian IGF-I mRNA levels than F×C ewes. Similarly, FFL IGF-II and estradiol concentrations were greatest in B-cross ewes and lowest in C ewes. Based on studies with human granulosa cells that show a stimulatory effect of IGF-II on estradiol production (Kamada et al., 1992; Barreca et al., 1993), it is possible that increased concentrations of IGF-II in FFL is responsible for increased FFL estradiol concentrations. Thus, genetic differences may exist in the regulation of follicular steroidogenesis and ovulation rate in sheep.

Which cell type(s) within the ovary contribute to the IGF-I mRNA detected in the whole ovary samples of the present study is uncertain but likely includes stromal cells, fibroblasts and cells of preantral follicles based on a preliminary report by Leeuwenberg et al., (1993) using in situ hybridization. These investigators found that IGF-I mRNA was present throughout the ovine ovary including the membrana granulosa but at low and constant amounts during an estrous cycle. Results similar to the present study were obtained in a study of human premenopausal ovaries

in which a solution hybridization/RNase protection assay detected IGF-I mRNA in whole ovaries but not granulosa cells (Hernandez et al., 1992). The source of the increased FFL IGF-II in B-cross ewes is uncertain but may include thecal and(or) granulosa cells because IGF-II mRNA has been localized in granulosa cells of human follicles (Geisthovel et al., 1989; El-Roeiy et al., 1993), in thecal cells of rat follicles (Hernandez et al., 1990), and in follicular walls of sheep follicles (present study). However, defining the roles of IGF-II vs IGF-I in regulating follicular growth in sheep will require further study.

The IGFBP have also been implicated as potential regulators of follicular development (Hammond et al., 1991; Giudice, 1992). In particular, the IGFBP in the ovary seem to play an important role in regulating the biological activity of IGF (Hammond et al., 1991; Giudice, 1992). For example, IGFBP-3 and IGFBP-2 inhibit FSH-induced estradiol synthesis in cultured rat granulosa cells by sequestering IGF-I (Ui et al., 1989; Bicsak et al., 1990). In addition, FFL levels of IGFBP-2 as well as other lower-molecular-weight IGFBP (but not IGFBP-3), assessed by ligand blotting, decrease as follicles develop in pigs (Mondschein et al., 1991; Howard and Ford, 1992; Echternkamp et al., 1994b) and cattle (Echternkamp et al., 1994a). Similarly, we observed significantly lower total IGFBP activity and lower activity of three forms of IGFBP (IGFBP-2, 27 to 29, and 24 kDa) in large follicles than in small follicles; genotype had no effect on total IGFBP activity in FFL (as assessed by both exchange assay and ligand blot). This decrease in total IGFBP activity was not a result of a decrease in IGFBP-3, but rather a decrease in activity of the lower-molecular-weight IGFBP, including IGFBP-2, as reported previously for pigs (Mondschein et al., 1991; Howard and Ford, 1992) and cattle (Echternkamp et al., 1994a). The identities of the 27- to 29- and 24-kDa IGFBP were not determined in the present study, but previous studies using Western blotting indicate that the 29-kDa IGFBP is likely IGFBP-1 (McLellan et al., 1992; Delhanty and Han, 1993). Due to a lack of specific antibodies, the identity of the 24-kDa IGFBP in sheep has not been confirmed, but based on its molecular weight it is speculated to be IGFBP-4 (Gallaher et al., 1992; McLellan et al., 1992; Delhanty and Han, 1993). A decrease in intrafollicular IGFBP activity together with an increase in IGF-I concentrations as follicles develop is thought to provide a coordinated sequence of events that facilitate IGF-I/IGF-II bioavailability for support of follicular growth and granulosa cell differentiation (Hammond et al., 1991). Other data indicate that atresia in ovine follicles less than 2 mm in diameter is associated with an increase in IGFBP-like activity localized within the granulosa cells and follicular antrum (Monget et al., 1989). The cause of the decrease in FFL IGFBP activity as follicles develop is unknown but may involve estradiol and(or) IGF-I; we observed a negative correlation between FFL estradiol concentrations and levels of IGFBP-2, 27 to 29 kDa, and 24-kDa IGFBP ($r = -.50$, $-.34$, and $-.26$, respectively, $P < .05$). Similarly, FFL IGF-I concentrations were negatively correlated with IGFBP-2, 27 to 29 kDa, and 24 kDa IGFBP ($r = -.74$, $-.56$, and $-.30$, respectively, $P < .05$). In contrast, FFL estradiol and IGFBP-3 levels were not correlated ($r = .17$, $P > .10$), whereas FFL levels of IGF-I and IGFBP-3 were positively correlated ($r = .44$, $P < .01$). Concentrations of IGF-II in FFL were not significantly correlated with FFL IGFBP activity or estradiol concentrations. Collectively, results from the present and previous studies (Mondschein et al., 1991; Samaras et al., 1992, 1994; Stanko et al., 1994) indicate that ovarian levels of IGFBP-2 and -3 are differentially regulated. Specifically, it seems that IGFBP-3 levels do not differ between small and medium or large follicles, whereas IGFBP-2 levels are greater in small than in medium or large follicles in sheep (present study), pigs (Mondschein et al., 1991; Echternkamp et al., 1994b), and cattle (Echternkamp et al., 1994a). The factors and(or) hormones that regulate ovarian levels of IGFBP-2 and -3 in sheep remain to be determined.

Implications

The regulation of folliculogenesis is a dynamic process in which insulin-like growth factor-I and -II (IGF-I and -II) likely play a role. Apparently there is a complex interplay between follicle-stimulating hormone, steroids (e.g., estradiol), growth factors, and other hormones that together control follicular development. The specific mechanism(s) whereby increased intraovarian production of estradiol and(or) IGF-II may alter folliculogenesis is unknown. In view of the stimulatory effects of IGF on steroidogenesis and mitogenesis in cultured granulosa and thecal cells, we propose that, in the presence of unchanging IGF binding protein activity, an increase in intrafollicular IGF-II in some genotypes of highly fecund sheep may stimulate development of more Graafian follicles.

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